Supplementary materials and methods

Plasmids

MSCV-MPLW515L-IRES-GFP retroviral vector was provided by Dr. Ross Levine (MSKCC). Glycerol stocks of pLKO.1 Human PIM1 shRNAs TRCN0000010115 (sh115) and TRCN0000010118 (sh118) and pLKO.1 mouse Pim1 shRNAs TRCN0000024382 (sh82) and TRCN0000024383 (sh83) were purchased from Dharmacon.

Retroviral transduction and bone marrow transplantation

Retroviral stocks of MSCV-MPLW515L-IRES-GFP were prepared by transient transfection of Plat-E cells and concentrated using centrifugation. To determine the requirement of Pim1 in MPLW515L-induced MF, BM cells from 5-fluorouracil (5-FU)-primed WT or Pim1KO mice were transduced with concentrated retroviruses expressing MPLW515L by two rounds of spin infection. Transduced bone marrow cells ($1x10^6$) were injected into retro-orbital veins of lethally irradiated (2 x 550 cGy) syngeneic recipient mice. Mice were maintained on acidified water. To study the effects of TP-3654/Ruxolitinib treatment in MPLW515L-induced MF, BM cells from 5-fluorouracil (5-FU)-primed WT BALB/c mice (Jackson Laboratories; stock # 000651) were transduced with retroviruses expressing MPLW515L and transplanted into retro-orbital veins of lethally irradiated (2 x 450 cGy) BALB/c recipient mice. In some cases, BM cells ($1x10^6$) from Mx1Cre; $Jak2^{VF/VF}$ mice were directly transplanted into lethally irradiated C57BL/6 syngeneic recipient mice and the transplanted animals were utilized for drug study.

Cell proliferation and apoptosis assays

To assess the effects of TP-3654/Ruxolitinib on cell proliferation, cells were plated in 6-well plates and treated with DMSO, TP-3654, Ruxolitinib or TP-3654 plus Ruxolitinib. Fresh cell culture medium and drugs were added to the cells every day. Cell proliferation was assessed by viable cell counts over 5 days by trypan blue exclusion assay. For apoptosis assays, cells were

treated with the drugs for 72 hours. Cell apoptosis was determined by Annexin V staining and analyzed by flow cytometry. To assess synergy between TP-3654 and Ruxolitinib, combination indices were determined using the median-effect principle of Chou and Talalay (CompuSyn Software) [Supplementary Ref. 1].

Immunoblotting

Peripheral blood mononuclear cells (PBMC) and mouse BM cells were lysed by direct boiling in 2x sample buffer. PIM1 knockdown or drug-treated HEL cells were lysed with RIPA lysis buffer containing protease inhibitors. Immunoblotting was performed using indicated phospho-specific or total antibodies. The following antibodies were used. Cell Signaling Technology: p-STAT5 (Tyr694) (#4322), STAT5 (#94205), p-SMAD2 (Ser465/Ser467) (#18338), SMAD2 (#5339), p-S6RP (Ser235/236) (#2211), S6RP (#2217), p-4E-BP1(Thr37/46) (#2855), p-BAD(Ser112) (#9291), Pim1 (#54523), c-MYC (#9402), ERK2 (#9108); Santa Cruz Biotechnology: 4E-BP1 (#sc-9977); Abclonal: p-EIF4B (S422) (#AP0775); Sigma: β-Actin (#A5441).

Flow cytometry

Single-cell suspensions were prepared from BM and spleen, and red cells were lysed with red cell lysis solution. Cells were washed and resuspended in PBS plus 2% FBS and stained for 20 minutes on ice with directly conjugated (either PE or APC) monoclonal antibodies specific for CD41, Mac-1 and Gr-1. For LSK (Lin-Sca1+c-kit+) cells analysis, cells were stained for 30 to 60 minutes on ice with antibodies against c-Kit, Sca-1, Flk2 (CD135), CD34, CD16/32 (FcγR II/III), and antibodies against lineage (Lin) markers including CD3, CD4, CD8, CD19, B220, Gr-1, Ter119, and IL-7R (CD127). All antibodies were purchased from eBioscience or BioLegend. Flow cytometry was performed with an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc.).

Colony-forming assays

Mice BM cells were plated (2 X10^4/dish) in duplicates in cytokine-supplemented complete methylcellulose medium (MethoCult M3434; StemCell Technologies). Granulocyte-macrophage colony-forming units (CFU-GM) colonies were scored on day 7. Human CD34+ cells were isolated from peripheral blood of healthy controls and MF patients using magnetic-activated cell isolation kit (Miltenyi Biotech). CD34+ cells were plated (1 X10^3/dish) in duplicates in cytokine-supplemented complete methylcellulose medium (MethoCult H4034; Stem Cell Technologies) in the presence of DMSO or TP-3654/Ruxolitinib. Colonies were scored on day 14.

RNA-sequencing analysis

LSK cells were sorted from Jak2$^{VF/VF}$ mice treated with vehicle or TP-3654 at 6 weeks after treatment using a FACS Aria II. Total RNA was extracted from LSK cells using RNeasy Micro kit (Qiagen). RNA sequencing was performed using NextSeq 500 High Output Kit and NextSeq 500 sequencing instrument (Illumina). RNA-seq data alignment was performed using UCSC mm10 reference genome with Bowtie2. The read counts and differential analysis were done using GenomicAlignments and DESeq2. P-adjusted value of <0.05 with a fold change of 1.5 was considered as significant change in gene expression. RNA-sequencing data generated in this study are deposited in the NCBI GEO repository under the accession number GSE183467.

Enzyme-linked immunosorbent assay (ELISA)

TGF-β1 levels in the serum of mice were determined using TGF-β1 ELISA kits (R&D Systems) according to the manufacturer's protocols.

MSC culture and immunofluorescence staining

Mesenchymal stromal cells (MSCs) were generated from WT mice as previously described [38]. For immunofluorescence staining, MSCs were grown on cover slips. MSCs were incubated with

TGF-β1 (50ng/ml) in the absence or presence of TP-3654 (2μM) for 72 hours. Collagen staining was performed with un-conjugated Abs against collagen I or collagen III (Abcam). Secondary staining was done using TRITC goat anti-rabbit antibody (Jackson ImmunoResearch). Fluorescence was visualized using Zeiss LAM 710 Confocal microscope.

Immunostaining

Immunostaining for p-S6RP, p-BAD, Snail and αSMA were performed as previously described [39]. Anti-pS6RP (Rabbit, 1:100, Cell Signaling), anti-pBAD (rabbit, 1:100, Cell Signaling), anti-SNAIL (rabbit, 1:100, Abclonal), and anti-αSMA (rabbit, 1:250, Abclonal) were used for staining. Secondary staining was done using TRITC goat anti-rabbit antibody (1:200 dilution) (Jackson ImmunoResearch). Mounting was done with VECTASHIELD mounting medium with DAPI (H-1200, Vector Laboratories). Fluorescence was visualized using Zeiss LAM 710 confocal microscope. Data were analyzed using Image J software (Image J).

Blood and tissue analysis

Peripheral blood counts were determined using Hemavet 950FS (Drew Scientific). For histopathologic analysis, mouse tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (4 μm) were stained with reticulin stain.

Supplementary figure legends

Supplementary Fig. 1. PIM1 expression is significantly elevated in hematopoietic cells of MF patients and Jak2V617F mice. A-B Elevated expression of PIM1 protein in the PBMC and BM of MF patients as compared to healthy controls. β-actin served as a loading control. **C** Immunoblot showing increased Pim1 protein expression in the BM of heterozygous ($Jak2^{VF/+}$) and homozygous Jak2V617F ($Jak2^{VF/VF}$) mice compared with control mice. **D** Immunoblot showing increased Pim1 protein expression in MPLW515L mice BM cells compared to control mice BM. **E** Immunoblot showing increased Pim1 protein expression in BA/F3 cells expressing JAK2V617F, MPLW515L or CALR del52 mutant compared to wild type JAK2 expressing BA/F3 cells. β-actin or Erk2 served as a loading control.

Supplementary Fig. 2. Knockdown of PIM1 significantly reduces proliferation of JAK2V617F-positive hematopoietic cells. A-D Knockdown of PIM1 (PIM1 KD) significantly inhibited proliferation of JAK2V617F-positive HEL, UKE-1 and BA/F3-EpoR-JAK2V617F cells but did not affect the proliferation of BA/F3-EpoR cells (n=3). Immunoblot showing effective knockdown of PIM1 in respective cell lines. β-actin served as a loading control. (*p<0.05; **p<0.005, ***p<0.0005; Student's t-test).

Supplementary Fig. 3. Transduction efficiency of retrovirus expressing MPLW515L. A Histograms showing flow cytometric analysis of GFP expression in BA/F3 cells infected with unconcentrated retrovirus expressing MSCV-MPLW515L-IRES-GFP. **B** Representative histograms of flow cytometric analysis of GFP expression in the BM of transplanted mice receiving WT and Pim1KO BM cells transduced with concentrated MPLW515L retrovirus. Bar graphs showing percentage of GFP-positive cells in the BM of transplanted mice. Data are

represented as mean ± SEM (n=3). Student's t test (unpaired two-tailed) was used to compare between 2 groups of mice (*p<0.05).

Supplementary Fig. 4. Genetic deletion of Pim2 does not prevent the development of myelofibrosis in MPLW515L mouse model. A Peripheral blood WBC, NE, RBC and PLT counts were assessed at 12 weeks after transplantation (n=6-10). **B** Spleen size/weight in WT; MPLW515L and Pim2KO; MPLW515L mice (n=6-10). Note that spleen weight was not significantly altered in Pim2KO; MPLW515L mice compared to WT; MPLW515L mice. Student's t test (unpaired two-tailed) was used to compare between two groups of mice (*p<0.05). **C** Reticulin staining show extensive fibrosis in the BM of both WT; MPLW515L mice and Pim2KO; MPLW515L mice at 12 weeks after transplantation. Scale bars, 15μm.

Supplementary Fig. 5. Treatment of TP-3654 induces significant apoptosis in JAK2V617F mutant hematopoietic cells. A BA/F3-EpoR cells expressing JAK2 WT were treated with vehicle or TP-3654 and cell proliferation was assessed by viable cell counts over 5 days. Data from three independent experiments are shown in bar graphs as mean ± SEM. **B** Apoptosis in BA/F3-EpoR cells after treatment with TP-3654 for 3 days was assessed by Annexin V/propidium iodide (PI) staining followed by flow cytometric analysis. Representative dot plots and bar graphs show that treatment of TP-3654 did not cause significant apoptosis in wild-type JAK2 expressing BA/F3-EpoR cells. **C** Representative dot plots of the flow cytometric analysis of the percentage of apoptotic cells in BA/F3-EpoR-JAK2V617F cells treated with TP-3654, Ruxolitinib and TP-3654/Ruxolitinib combination. Treatment of TP-3654, Ruxolitinib and TP-3654/Ruxolitinib combination resulted in significant apoptosis in BA/F3-EpoR-JAK2V617F cells. **D** Annexin V/propidium iodide (PI) staining and flow cytometric analysis was performed to measure apoptosis in JAK2V617F-positive HEL cells after treatment with DMSO, TP-3654, Ruxolitinib and TP-3654/Ruxolitinib combination. Representative dot plots are shown in the left.

Bar graphs (in the right) showing percentage of apoptotic cells as mean ± SEM (n=3; *p<0.05; **p<0.005; ***p<0.0005; Student's t test).

Supplementary Fig. 6. Effects of TP-3654/Ruxolitinib treatment on healthy control hematopoietic progenitor colony formation. Healthy control CD34+ cells were plated in complete methylcellulose medium supplemented with cytokines in the presence of DMSO, TP-3654, Ruxolitinib and TP-3654/Ruxolitinib combination. Data are represented in bar graphs as mean ± SEM (n= 4). One-way ANOVA was used for comparisons of all 4 groups of treatment, and the Student's t test (unpaired two-tailed) was used to compare between 2 groups of drug treatment (***p<0.0005, ****p<0.00005).

Supplementary Fig. 7. Pim inhibitor TP-3654 is effective against Ruxolitinib-resistant JAK2V617F mutant hematopoietic cells. A Immunoblot showing no significant change in STAT5 phosphorylation in Ruxolitinib-resistant BA/F3-EpoR-JAK2V617F-Rux^R cells upon Ruxolitinib treatment. β-actin served as a loading control. **B** Ruxolitinib (0.25-1μM) treatment did not inhibit proliferation of BA/F3-EpoR-JAK2V617F-Rux^R cells (n=3). **C-D** In vitro treatment of BA/F3-EpoR-JAK2V617F-Rux^R cells with TP-3654 (0.25-1μM) alone or in combination with Ruxolitinib (0.25- 0.5 μM) showed significant reduction in cell proliferation (n=3). **E-F** RT-qPCR and immunoblot analyses showing significant increase in Pim1 expression in BA/F3-EpoR-JAK2V617F cells compared with BA/F3-EpoR cells. Pim1 expression was further increased in Ruxolitinib-resistant BA/F3-EpoR-JAK2V617F-Rux^R cells as compared to BA/F3-EpoR-JAK2V617F cells. The mRNA expression was normalized by *Gapdh* (n=4). (*p<0.05; **p<0.005; ***p<0.0005; Student's t-test).

Supplementary Fig. 8. Effects of Pim1 overexpression on Ruxolitinib sensitivity in Ba/F3-EpoR-JAK2V617F cells. A BA/F3-EpoR-JAK2V617F cells were transduced with lentiviruses

expressing vector or Pim1 WT and the infected cells were selected using puromycin. Immunoblot showing overexpression of exogenous Pim1 WT. **B** BA/F3-EpoR-JAK2V617F cells expressing vector or Pim1 WT were treated with DMSO or Ruxolitinib at indicated concentrations for 4 days and cell proliferation was assessed. Note that overexpression of Pim1 significantly reduced the sensitivity of Ruxolitinib in BA/F3-EpoR-JAK2V617F cells. Data are represented in bar graphs as mean ± SEM. (*p<0.05; **p<0.005; ***p<0.0005; Student's t-test).

Supplementary Fig. 9. Effects of TP-3654/Ruxolitinib treatment on wild type mice. To assess the in vivo effects of TP-3654/Ruxolitinib treatment on control animals, wild type mice were treated with vehicle, TP-3654 (150mg/kg/per day), Ruxolitinib (60mg/kg/per day) or TP-3654 (150mg/kg/per day) plus Ruxolitinib (60mg/kg/per day) for 3 weeks by oral gavage. **A** Body weights of wild type mice following treatment with TP-3654, Ruxolitinib and TP-3654/Ruxolitinib combination. **B** Peripheral blood WBC, neutrophil (NE), platelet (PLT), RBC, hemoglobin (Hb), and hematocrit (HCT) counts in wild type mice following treatment with TP-3654, Ruxolitinib and TP-3654/Ruxolitinib combination. One-way ANOVA was used for comparisons of all 4 groups of treated mice, and the Student's t test (unpaired two-tailed) was used to compare between two groups (*p<0.05).

Supplementary Fig. 10. Suppression of Pim1 activity alters cell signaling in MPN cells. A PBMC obtained from MF patients were treated with DMSO or TP-3654 (TP), Ruxolitinib (Rux) or TP plus Rux at indicated concentrations for 6 hours. Immunoblotting was performed using phospho-specific or total antibodies as indicated. Treatment of TP-3654 alone significantly reduced phosphorylation of EIF4B, S6RP, 4E-BP1, BAD and SMAD2 and reduced the expression of c-MYC in MF PBMC. Combined treatment of TP-3654 with Ruxolitinib caused greater reduction of phosphorylation or expression of these signaling proteins. **B** BM cells obtained from $Jak2^{VF/VF}$ mice following in vivo treatment with vehicle, TP-3654, Ruxolitinib or TP-

3654/Ruxolitinib combination were subjected to immunoblotting using phospho-specific or total antibodies as indicated. β-Actin was used as a loading control.

Supplementary Fig. 11. Effects of PIM1 knockdown on signaling in HEL cells. JAK2V617F-positive human HEL cells were transduced with lentiviral scramble shRNA (control) or PIM1 shRNAs and selected using puromycin. Immunoblot analyses show decreased phosphorylation of S6RP, 4E-BP1 and BAD, and reduced expression of c-MYC upon PIM1 knockdown (PIM1 KD 1 and PIM1 KD 2) in HEL cells. β-Actin was used as a loading control.

Supplementary Fig. 12. Effects of TP-3654 treatment on PIM1 expression in HEL cells. JAK2V617F-positive human HEL cells were treated with TP-3654 (1 and 2 μM) for 24 hours. The RT-qPCR analyses show increased PIM1 and PIM2 expression in HEL cells treated with TP-3654. The mRNA expression was normalized by HPRT. Data are shown in bar graphs as mean ± SEM (n =4; *p<0.05; **p<0.005; Student's t-test).

Supplementary Fig. 13. Immunohistochemistry on BM sections from homozygous Jak2V617F mice treated with TP-3654/Ruxolitinib. A-B Representative immunofluorescence images showing phospho-S6RP and phospho-BAD immunostaining in the BM sections of $Jak2^{VF/VF}$ mice treated with vehicle, TP-3654, Ruxolitinib or TP-3654/Ruxolitinib combination. pS6RP and pBAD in yellow and DAPI in blue. Scale bars, 15μm.

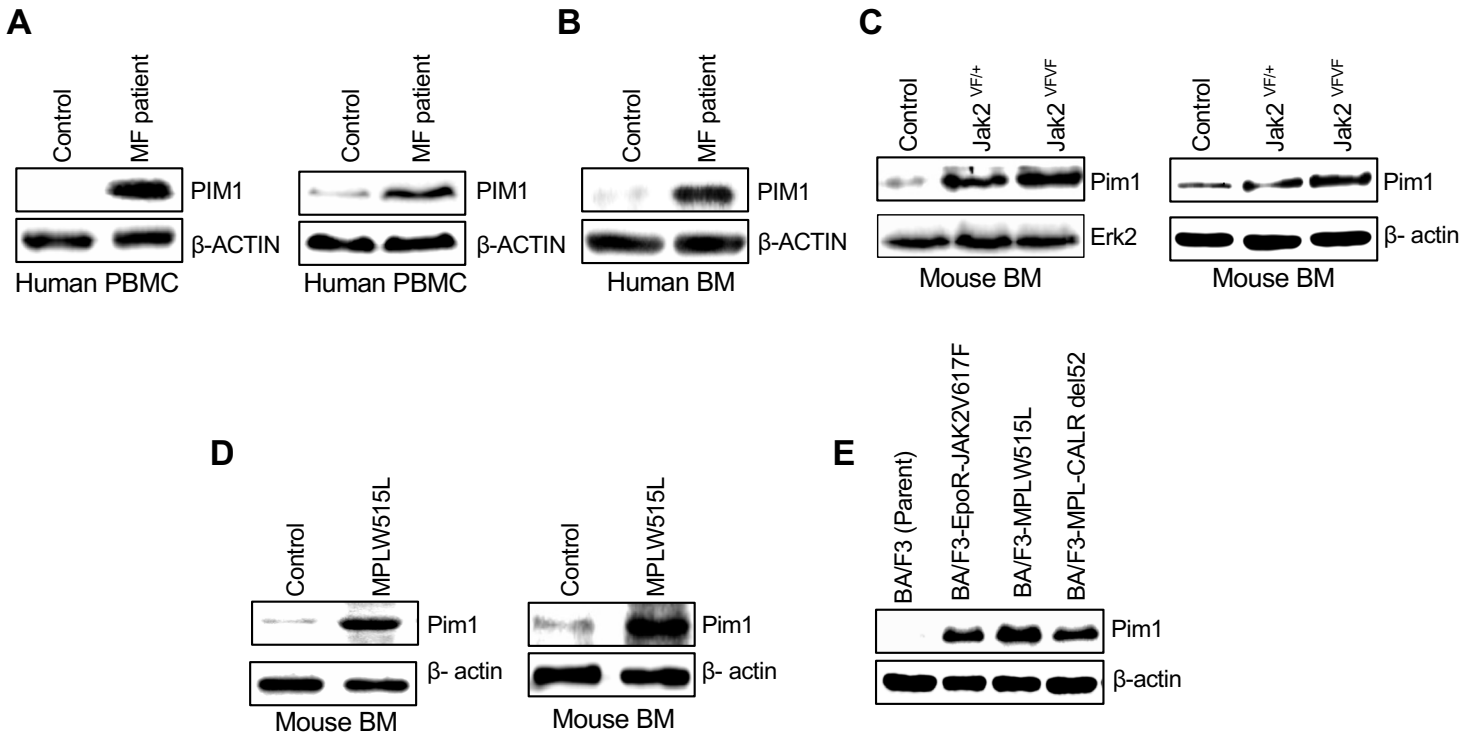
Supplementary Fig. 14. Effects of TP-3654 on Collagen expression in BM MSC. A-B Mouse BM derived MSCs were treated with DMSO, TP-3654 alone (1-2μM), TGF-β1 (50ng/ml) and TGF-β1 (50ng/ml) plus TP-3654 (2μM). Stimulation with TGF-β1 (50ng/ml) significantly increased Collagen I (**A**) and Collagen III (**B**) expression in BM MSC. Treatment of TP-3654

significantly reduced TGF-β1 induced Collagen I and III expression in BM MSC. The mRNA expression was assessed by RT-qPCR and normalized by Hprt. Data are shown in bar graphs as mean ± SEM (n =4; *p<0.05; Student's t-test).

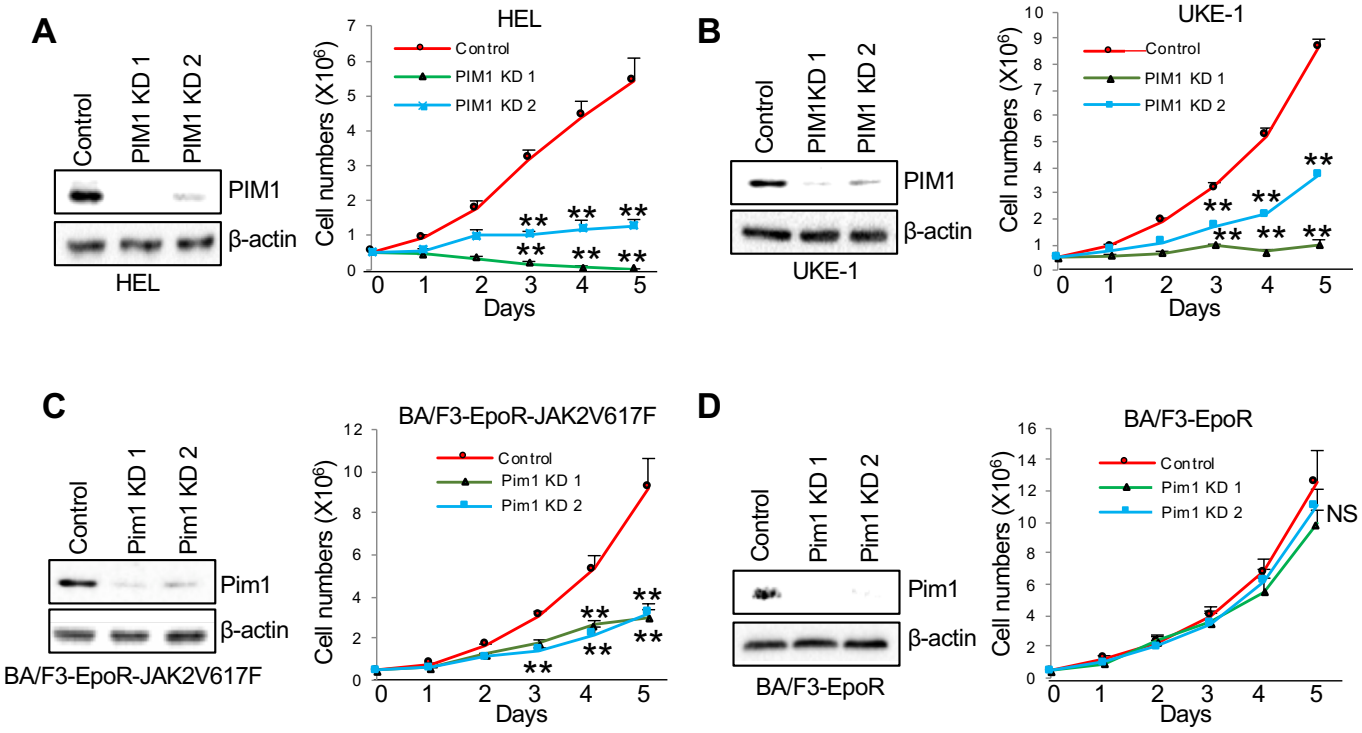
Supplementary Fig. 15. TP-3654 treatment reduces Collagen expression in BM MSC. A-B Immunofluorescence images showing increased expression of Collagen I (**A**) and Collagen III (**B**) in BM MSCs stimulated with TGF-β1 (50ng/ml). TP-3654 (2μM) treatment significantly reduced TGF-β1 induced Collagen I and III expression. Collagen I (green), Collagen III (red) and DAPI (blue); scale bars, 50μm.

Supplementary Reference

1. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010;70:440-446.



Supplementary Figure 1



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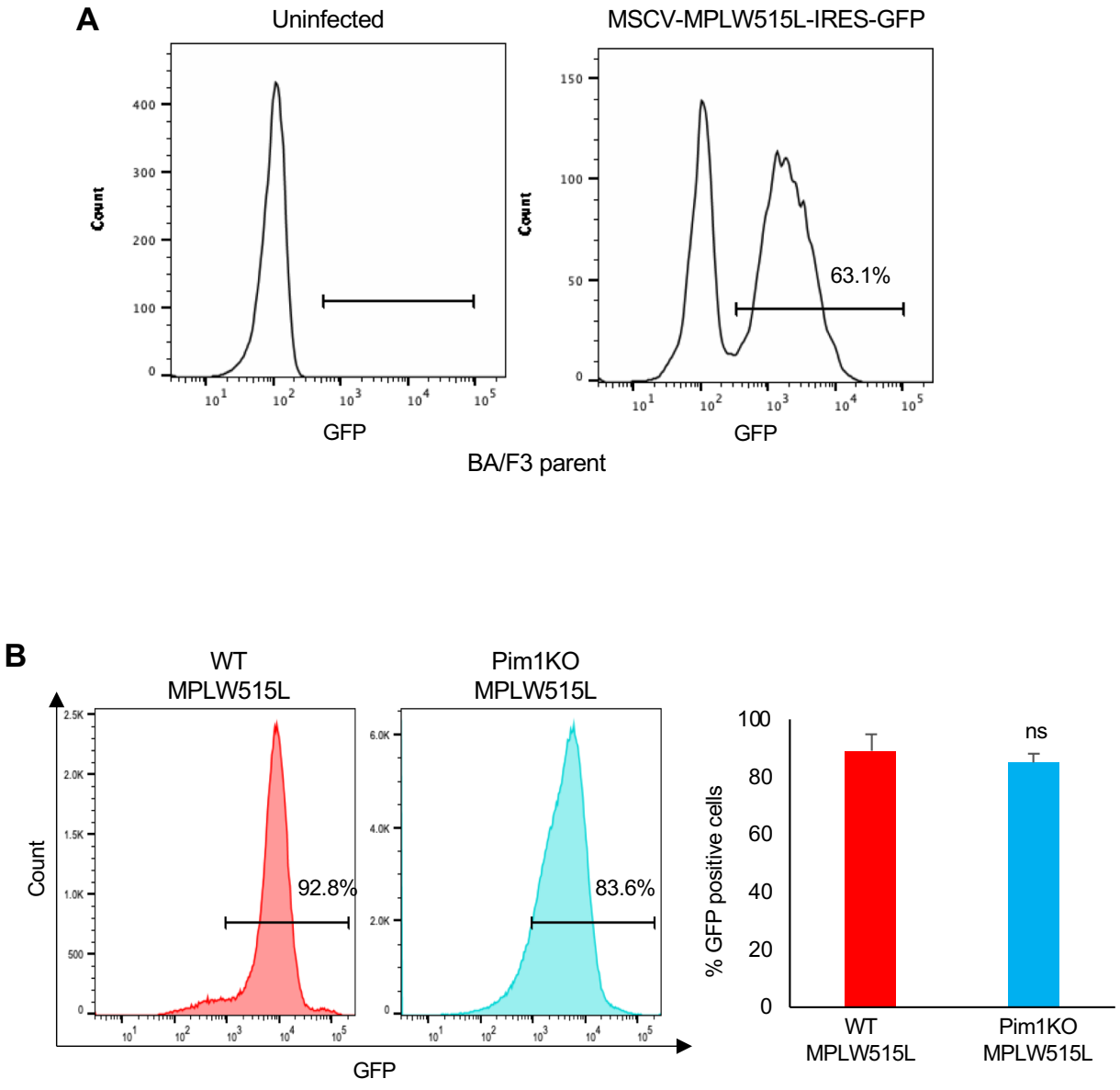

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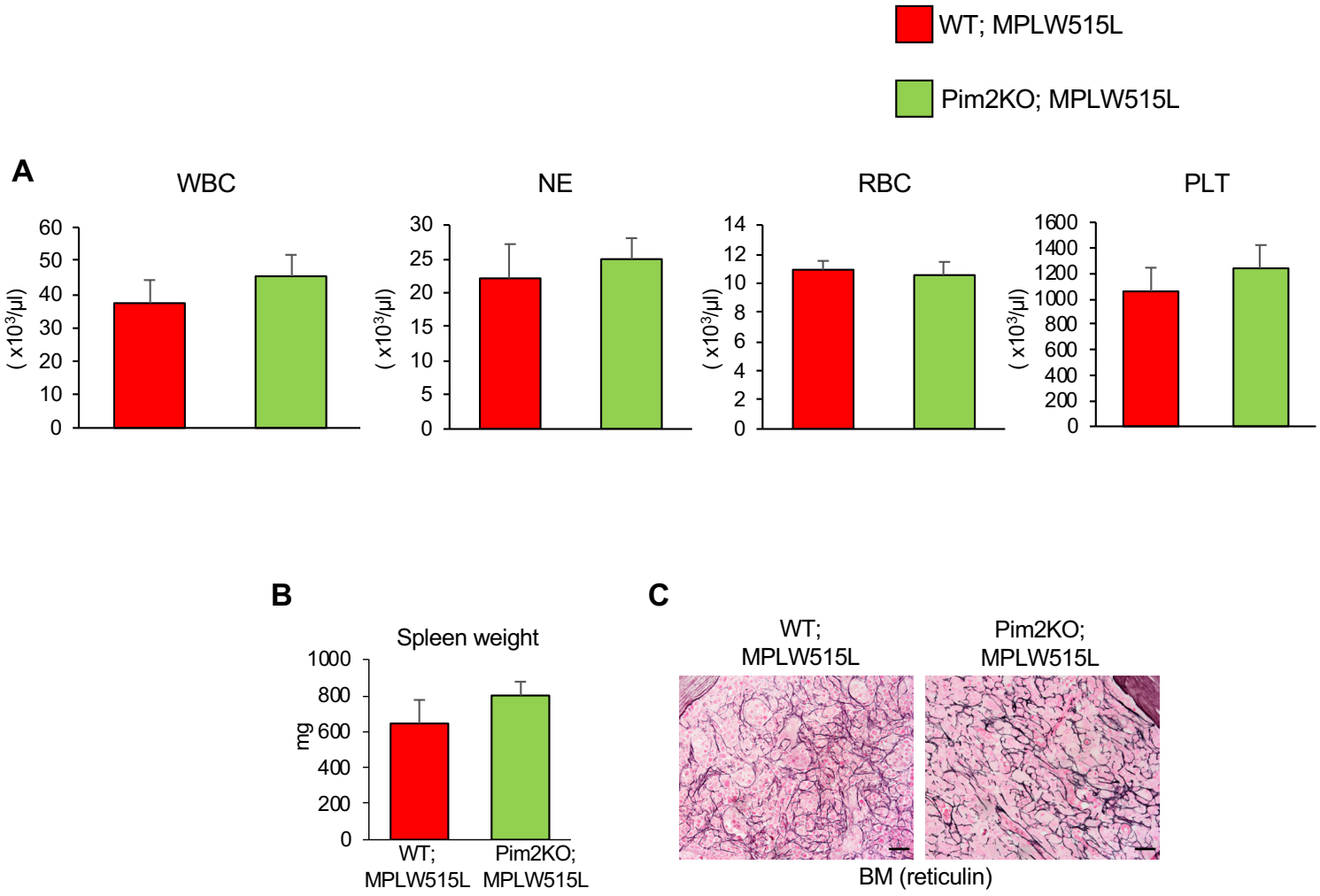

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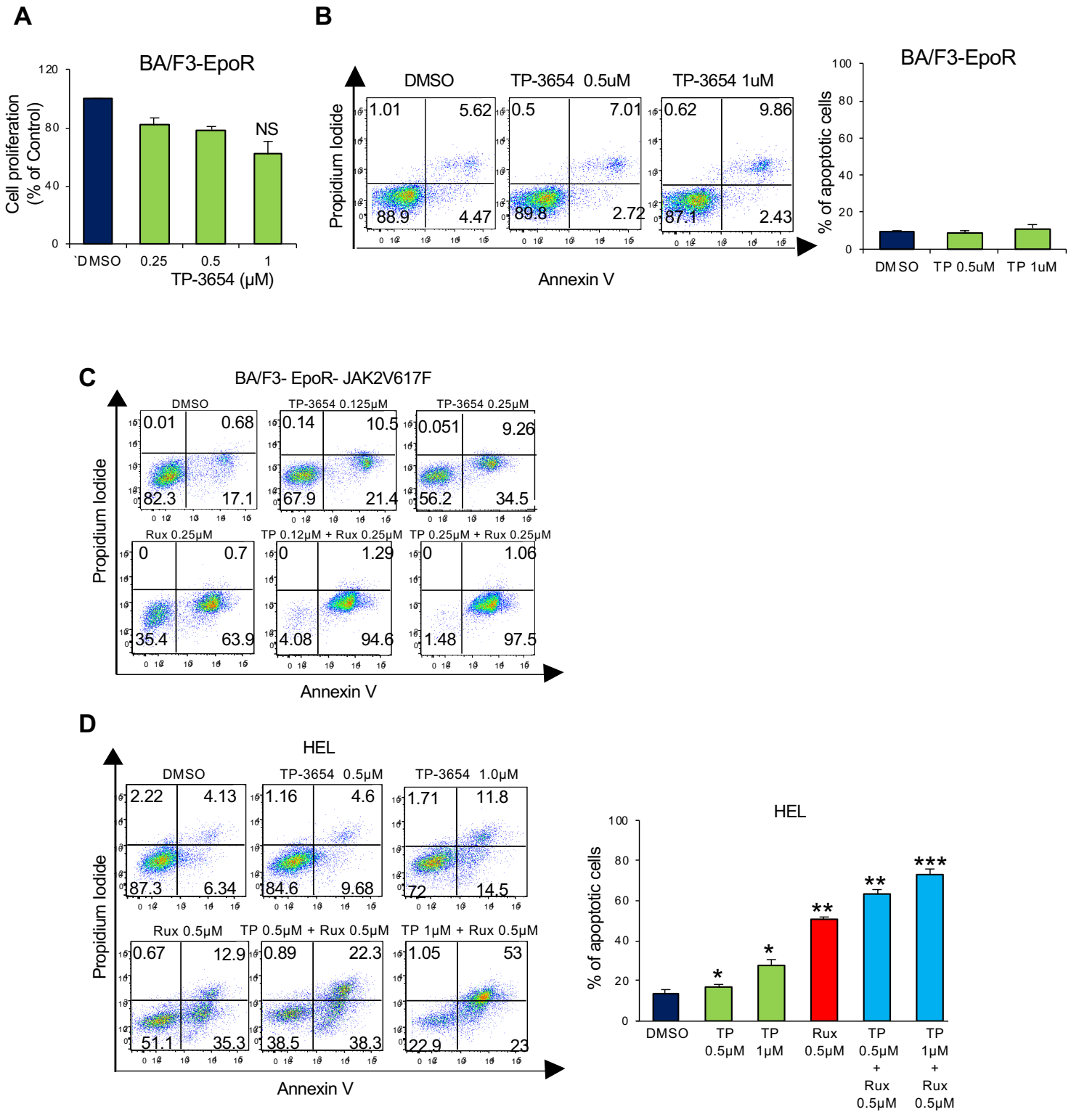

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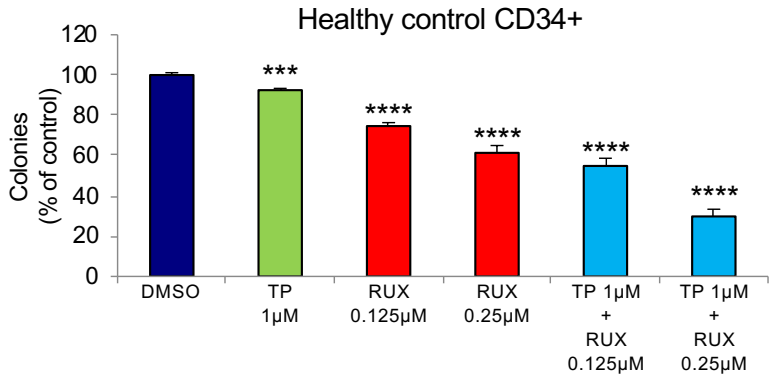

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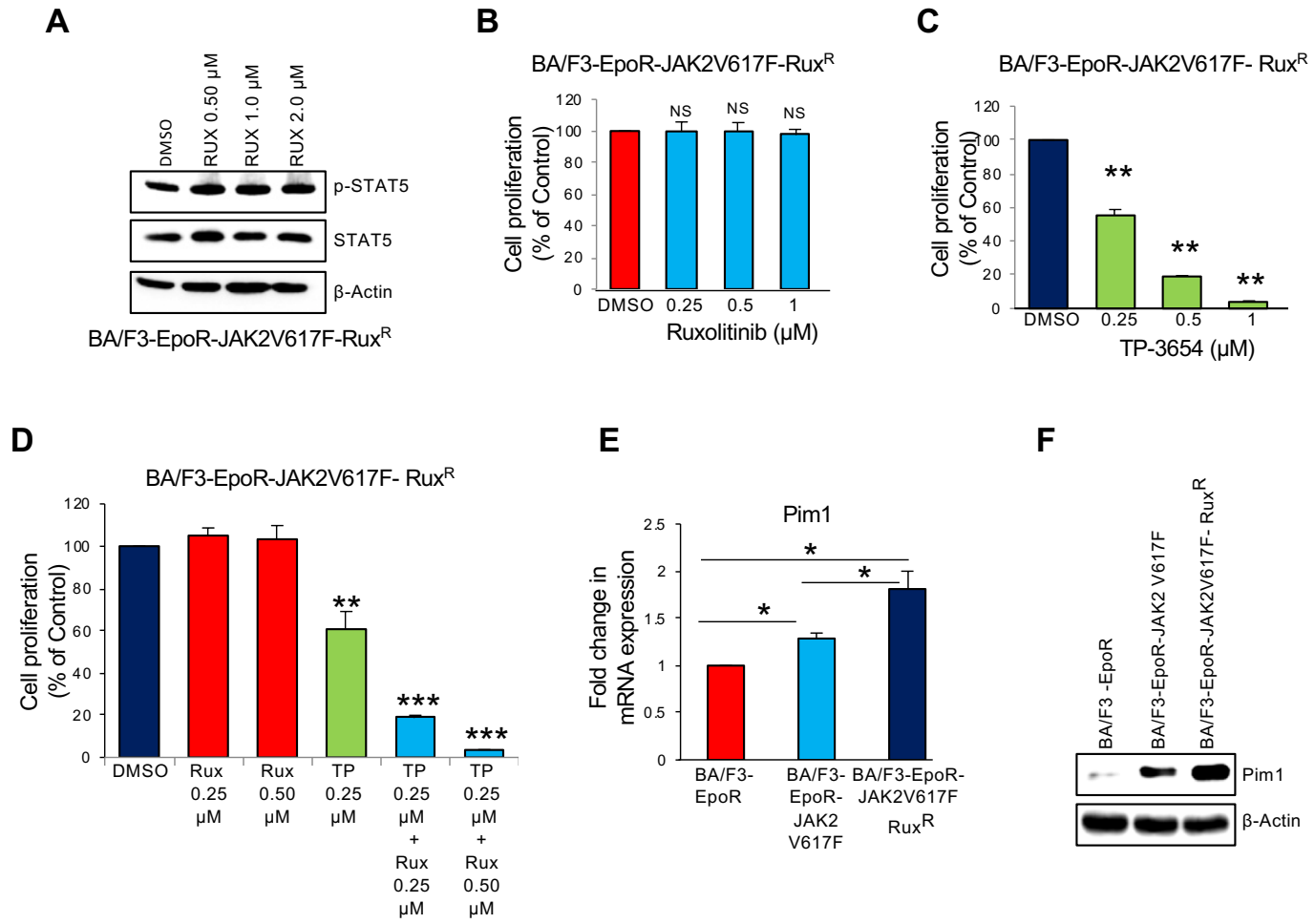

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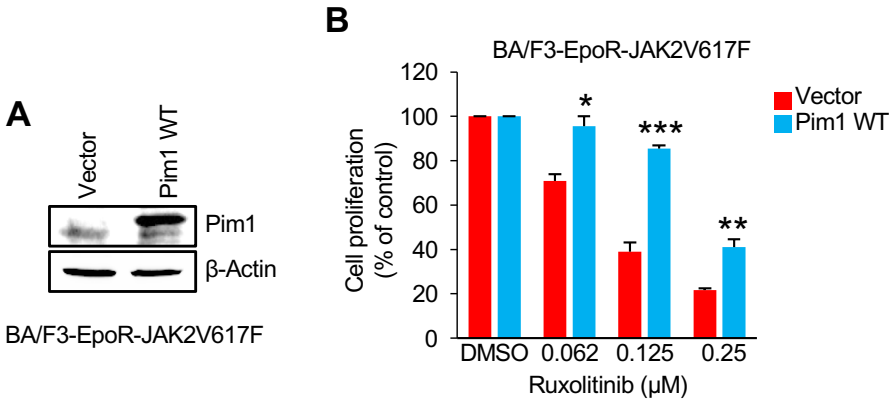

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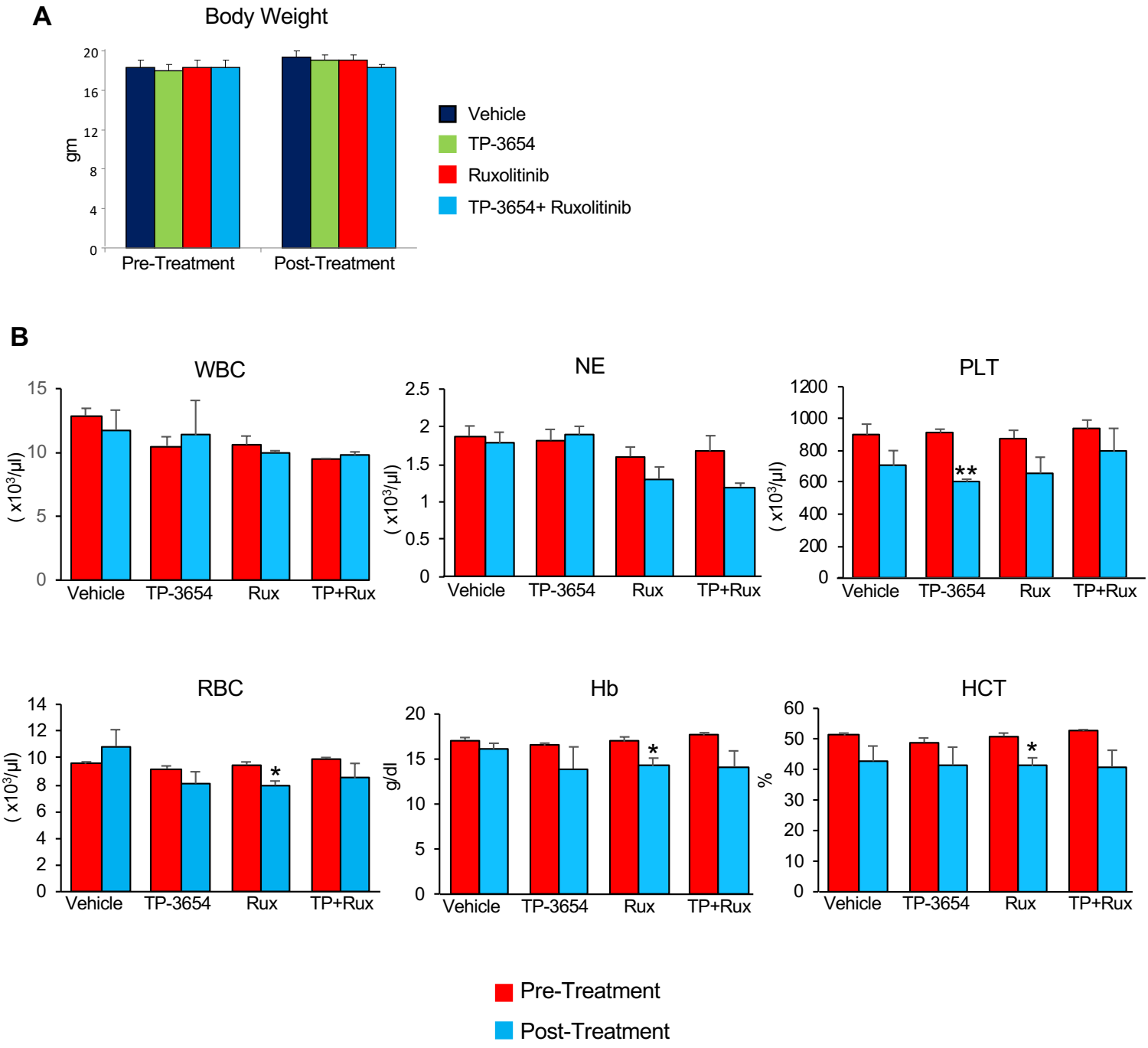

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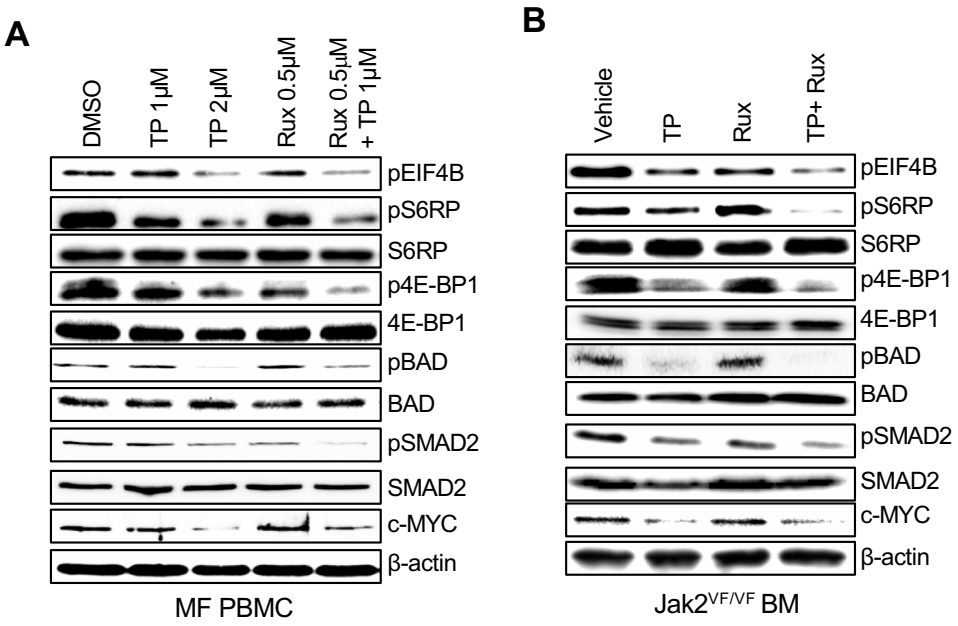

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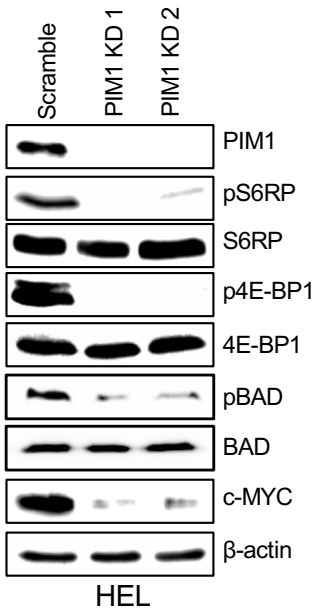

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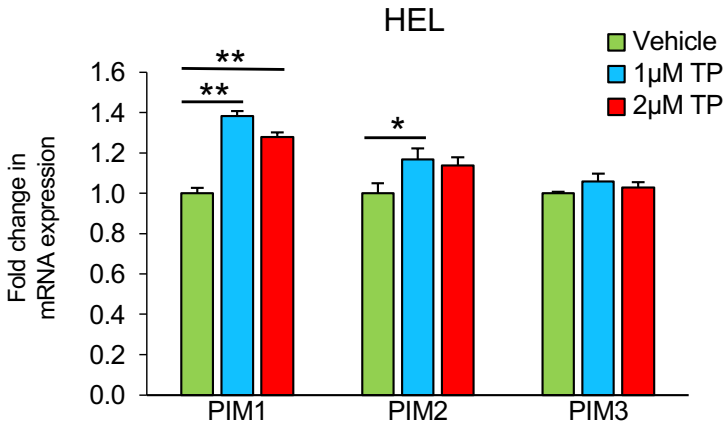

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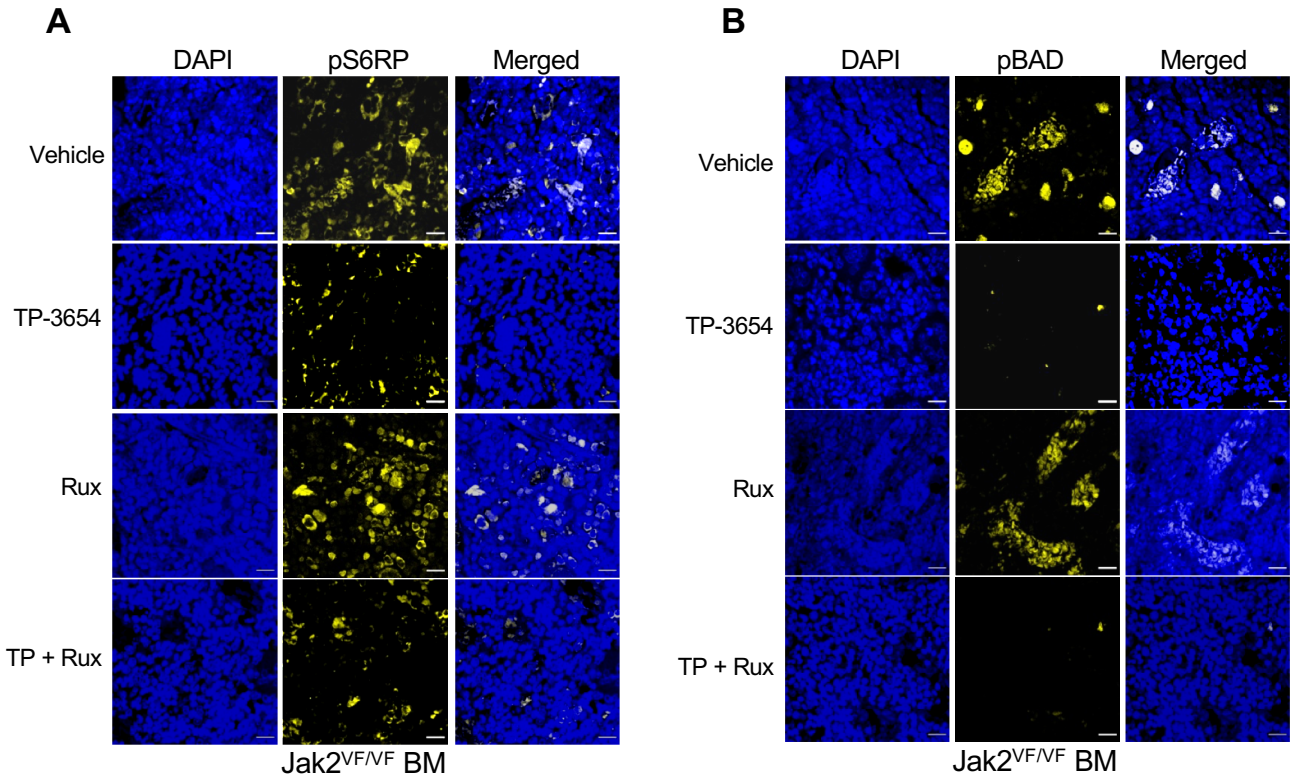

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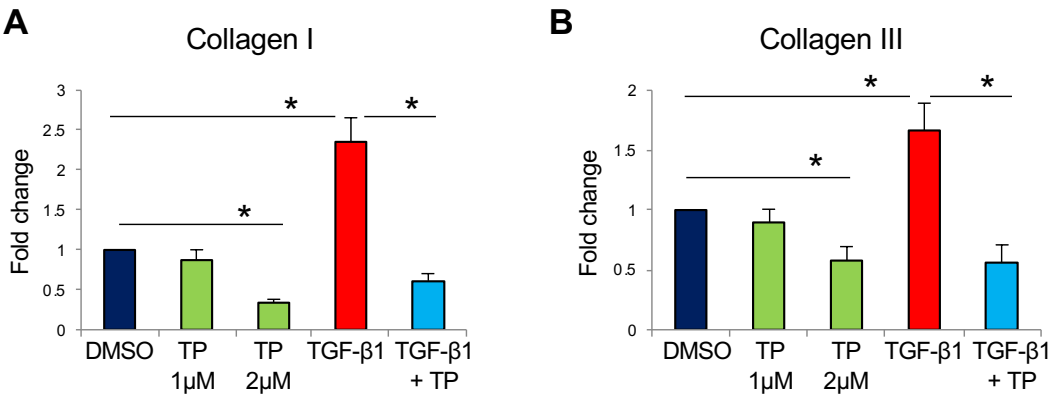

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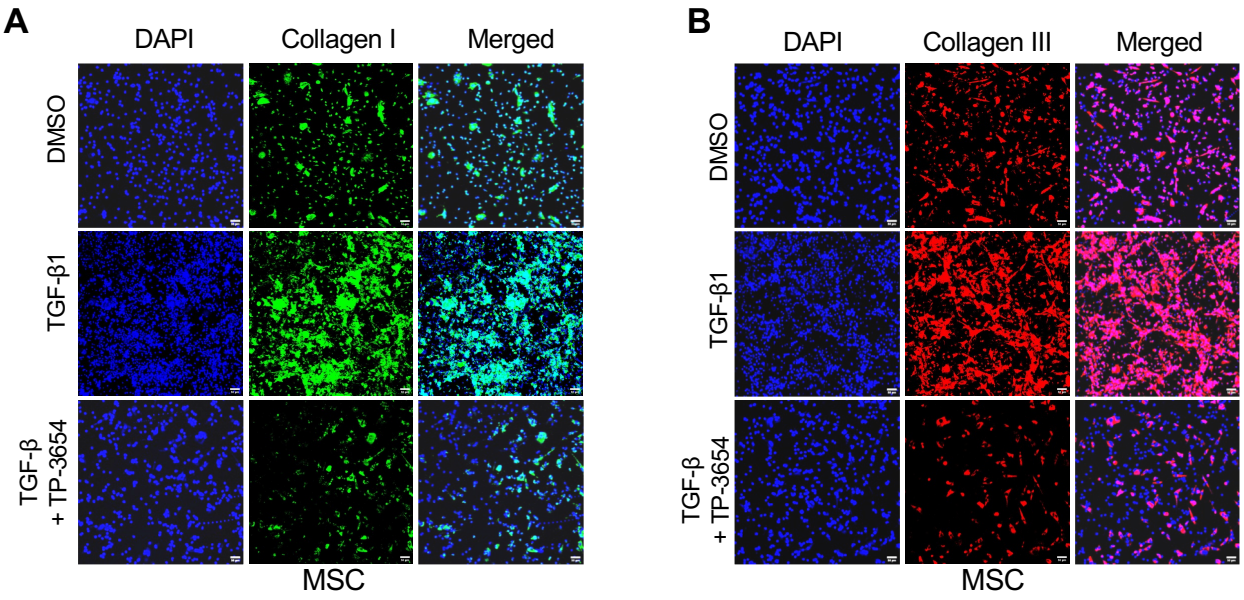

Supplementary Figure 15

Supplementary Table 1. Primers used for real-time quantitative PCR

mouse *Col1a1* Forward	TGGTCCCTCTGGAAATGCTG
mouse *Col1a1* Reverse	AACTTCACCAGGACGTCCAG
mouse *Col3a1* Forward	TGACAGAGGAGAAACTGGCC
mouse *Col3a1* Reverse	GCCATTAGAGCCACGTTCAC
mouse *Pim1* Forward	CAGTCTACACGGACTTTGATGG
mouse *Pim1* Reverse	CGCAGACCATGTCATAGAGC
mouse *18S* Forward	CGCCGCTAGAGGTGAAATTC
mouse *18S* Reverse	TTGGCAAATGCTTTCGCTC
mouse *Hprt1* Forward	CAACGGGGGACATAAAAGTTATTGGTGGA
mouse *Hprt1* Reverse	TGCAACCTTAACCATTTTGGGGCTGT
mouse *Gapdh* Forward	ACTCCACTCACGGCAAATTC
mouse *Gapdh* Reverse	TCTCCATGGTGGTGAAGACA
human *PIM1* Forward	GAGGTTGGGATGCTCTTGTC
human *PIM1* Reverse	GGTCCTTCTCCACGTGTTTG
human *PIM2* Forward	GCACTGCTATGGAAAGTGGGT
human *PIM2* Reverse	ATGGACAACTCCACGGGAATG
human *PIM3* Forward	AAGGACGAAAATCTGCTTGTGG
human *PIM3* Reverse	CGAAGTCGGTGTAGACCGTG
human *HPRT1* Forward	TGCAGACTTTGCTTTCCTTGGTCAGG
human *HPRT1* Reverse	CCAACACTTCGTGGGGTCCTTTTCA
human *GAPDH* Forward	GAGTCAACGGATTTGGTCGT
human *GAPDH* Reverse	GACAAGCTTCCCGTTCTCAG
human *18S* Forward	CGCCGCTAGAGGTGAAATTC
human *18S* Reverse	TTGGCAAATGCTTTCGCTC